In Situ Observation of Spherical DNA Assembly in Water and the Controlled Release of Bound Dyes

Kazunori Matsuura,* Kouzo Masumoto, Yuuko Igami, Tatsuro Fujioka, and Nobuo Kimizuka

Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, Moto-oka 744, Nishi-ku, Fukuoka 819-0395

Received March 31, 2007; Revised Manuscript Received June 27, 2007

Three strands of 30-mer oligodeoxyribonucleotides (ODNs) were designed to form three-way junctions that possess self-complementary sticky ends. The morphology of self-assembled ODNs in water was observed in situ by confocal laser scanning fluorescence microscopy. The three-way junctions self-assembled into spherical assemblies, in accordance with transmission and scanning electron microscopy. The size of nucleospheres was in the range of several tens of nanometers to micrometers, which varied depending on the concentration of ODNs and added salts. Fluorescence images of spherical ODN assemblies suggested that the nucleospheres possess multiwalled structures. The fluorescence of sodium 1-anilinonaphthalene-8-sulfonate in the presence of nucleospheres revealed that the interior of nucleospheres possesses polarity corresponding to that between methanol and ethanol. A dye-inclusion experiment showed that cationic and even anionic dyes were adsorbed to the interior of the nucleospheres. The dye-included nucleospheres released dyes by thermal dissociation or digestion of the constituent ODNs.

Introduction

In nature, a variety of functional capsule structures are formed by self-assembly. For example, cell membranes and spherical viruses are spontaneously formed by the self-assembly of constituent lipids and proteins.[1−2] Inspired by such biomolecular capsules, construction and control of artificial capsule structures have been active areas in molecular assembly. The first generation of synthetic supramolecular capsules goes back to the discovery of synthetic bilayer vesicles in 1977,[3] which opened the field of self-assembly in chemistry.[4] More recently, synthetic capsules have been developed by using designable molecular interactions, such as hydrogen bonding[5] or metal coordination.[6] Amphiphiles are now designed from a wide variety of molecules, as exemplified by rod−coil diblock copolymers that provide micro-sized hollow capsules.[7] Nolte et al. reported "giant amphiphiles" that consist of proteins and polystyrenes, which allow the organization of modified proteins.[8] Another approach in the self-assembly of hollow microcapsules has employed layer-by-layer adsorption of polyelectrolytes on template colloids.[9] However, the demand of biodegradable capsules is not satisfied by these pre-existing examples. It still remains difficult to obtain nano- to micron-sized capsules by the self-assembly of pure biomolecules.

Oligodeoxyribonucleotides (ODNs) have been attracting much attention as molecular components for the self-assembly of nanoarchitectures.[10,11] One of the pioneering works was achieved by Seeman et al., which included the development of designed DNA nanostructures such as the cube,[11a] the truncated octahedron,[11b] and DNA nanosheets.[11f] The self-assembly of ODNs has been applied to DNA computing,[12] molecular machines,[13] and the formation of nanogels.[14] ODNs also serve as scaffolds to direct functional arrays of metal nanoparticles,[15] proteins,[16] chromophores,[17] and sugars.[18]

We reported a simple one-pot strategy to prepare nano-sized spherical DNA assemblies by using the self-assembly of designed ODNs **1**, **2**, and **3** (Figure 1).[19] Mixing of the three designed 30-mer ODNs at equimolar concentrations in water affords DNA three-way junctions bearing self-complementary sticky ends. When these DNA three-way junctions consume all of the self-complementary sticky ends, nucleospheres are observed by transmission electron microscopy (TEM). However, as TEM images are obtained for dried specimens, it is required to prove their presence in aqueous solutions.

Recently, we reported in situ observation of the spherical structure of nucleospheres in aqueous solution by using confocal laser scanning fluorescence microscopy (CLSM).[20] This paper describes a detailed characterization of nucleospheres in water, together with their dye-inclusion properties.

Experimental Section

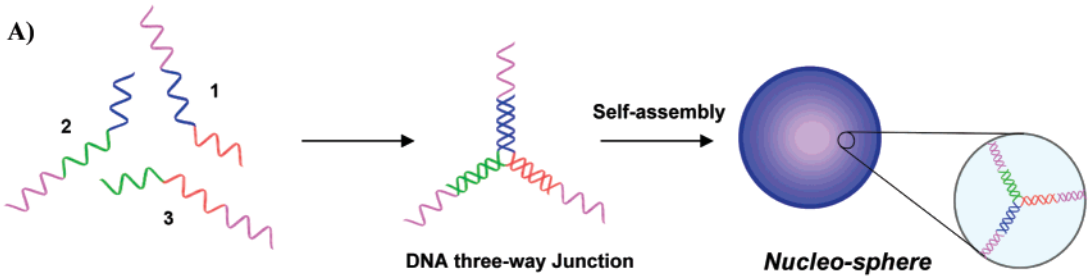
Materials. All types of synthetic ODNs, including 6-carboxyfluorescein (FAM)-labeled ODN (purified by high-performance liquid chromatography) were purchased from Espec Co., Ltd. as lyophilized powders and used without further purification. ODN solutions were prepared in 1 M NaCl (aq) and stored at −10 °C. The concentration of ODN was determined by UV absorbance at 260 nm. λ-DNA (48 502 bp) and salmon testes DNA (ca. 2000 bp) were purchased from Wako Pure Chemical Co., Ltd. and Sigma, respectively. Ethidium bromide (EB) was purchased from JANSSEN Co., Ltd. YOYO-1 iodide was purchased from Molecular Probe Co., Ltd. as 1 mM dimethyl sulfoxide solution. Exonuclease III was purchased from Promega Co., Ltd. A TaKaRa DNA Ligation kit, version 2, was purchased from TAKARA Shuzo Co., Ltd.

Typical Procedure for Construction of Nucleospheres. Equimolar aqueous solutions of ODNs **1**−**3** were mixed at room temperature ($[ODN]_{total}$ = 1−30 μM, [NaCl] = 0.5 M). After heating the mixture at 70 °C for 5 min, it was cooled spontaneously to 10 °C and then aged at this temperature for 12 h.

Transmission Electron Microscopy. TEM was conducted on a JEM-2010 instrument (JEOL) by the negative staining method. One drop of solutions containing nucleospheres was placed on a carbon-coated Cu-grid (Oken Co., Ltd.) at 10 °C and dried in vacuo. Subsequently, a drop of 0.2 wt % aqueous uranyl acetate was placed

* To whom correspondence should be addressed. Phone: +81 92 802 2833. Fax: +81 92 802 2838. E-mail: ma14tcm@mbox.nc.kyushu-u.ac.jp.

10.1021/bm070357w CCC: $37.00
Published on Web 08/17/2007



B)

1 5'-GGCGTGGTAGACCGCACTCGAAAAATTTTT-3'
2 5'-CGAGTGCGGTGACGATGCCTAAAAATTTTT-3'
2FAM 5'-CGAGTGCGGTGACGATFAMGCCTAAAAATTTTT-3'
3 5'-AGGCATCGTCCTACCACGCCAAAAATTTTT-3'
4 5'-GGCGTGGTAGACCGCACTCG-3'
5 5'-CGAGTGCGGTGACGATGCCT-3'
6 5'-AGGCATCGTCCTACCACGCC-3'
7 5'-GGCGTGGTAGACCGCACTCGGGGGGCCCCC-3'
8 5'-CGAGTGCGGTGACGATGCCTGGGGGCCCCC-3'
9 5'-AGGCATCGTCCTACCACGCCGGGGGCCCCC-3'
10 5'-GGCGTGGTAGACCGCACTCGATATATATAT-3'
11 5'-CGAGTGCGGTGACGATGCCTATATATATAT-3'
12 5'-AGGCATCGTCCTACCACGCCATATATATAT-3'

Figure 1. (A) Schematic illustration of the formation of nucleospheres by the self-assembly of DNA three-way junctions bearing self-complementary sticky ends. (B) DNA sequences used in this study. Sequences with identical colors are complementary to each other. Underlined sequences indicate self-complementary sticky ends.

on each of the grids and dried in vacuo (post-staining method). The Cu meshes were subjected to TEM observation with an acceleration voltage of 100 kV and magnifications of 50 000−100 000.

Scanning Electron Microscopy (SEM). SEM was conducted on a Hitachi S-5000 instrument. The unstained TEM specimen of nucleospheres on a carbon-coated Cu-grid was coated with platinum (ca. 7−9 nm: Hitachi E-1030 ion sputter, 10 mA, 10 Pa, 50 s). The Cu meshes were subjected to SEM observation with an accelerating voltage of 25 kV at a tilt angle of 40°.

Confocal Laser Scanning Microscopy. CLSM was conducted on an LSM 510 instrument (Carl Zeiss) equipped with an LP 505 filter (cut off < 505 nm). An aliquot (typically 30 μL) of aqueous nucleospheres was placed on a glass dish at 25 °C. The sample was stained by EB (100 μM) or YOYO-1 iodide (1 μM), when necessary. The dish was subjected to CLSM observations with excitation at 488 nm (Ar^+ laser) through an LP505 filter.

Inclusion of Dyes to Nucleospheres. Aqueous NaCl (0.5 M) solutions containing equimolar ODNs **1**−**3** ($[ODN]_{total}$ = 100 μM) and dyes (100 μM) were heated to 70 °C and kept at this temperature for 5 min. It was then cooled to 10 °C at a cooling rate of −0.33 °C min^{-1}, and aged at this temperature for 12 h. An aliquot (30 μL) of the solution was observed by CLSM with excitation at 543 nm (HeNe laser) through an LP505 filter. An aliquot (50 μL) of the solution was centrifuged at 10 000 rpm for 10 min. The amount of dye in the supernatant was determined by UV−vis spectroscopy.

Results and Discussion

CLSM Observation of Nucleospheres in Aqueous Solution. In order to observe nucleospheres by CLSM, nucleospheres were prepared from fluorescence-labeled ODNs. A T-base in the center of ODN **2** was labeled with the fluorescent dye FAM (**2^{FAM}**), as this position is expected to show the smallest influence on the self-assembly. Equimolar solutions of the three ODNs, **1**, **2^{FAM}**, and **3**, were mixed in 0.5 M aqueous NaCl ($[ODN]_{total}$ = 0.1−30 μM, at room temperature). The mixture was first heated to 70 °C with a heating rate of 1 °C/min, and was then incubated at 70 °C for 5 min. Succeedingly, the heated aqueous mixture was spontaneously cooled to room temperature, and incubated at 10 °C for 24 h.

In CLSM, spherical assemblies with an average diameter of 3.88 ± 1.03 μm were abundantly seen (Figure 2A, $[ODN]_{total}$ = 20 μM, at 25 °C). By taking a blurring effect[21] of ca. 0.3 μm into consideration, the actual size of the spherical assemblies is estimated to be about 3.3 μm. Non-labeled nucleospheres were also constructed from ODNs (**1**+**2**+**3**). To observe the aggregate structures in CLSM, they were stained with fluorescent intercalators YOYO-1 iodide and EB. EB was added to the solution of nucleospheres at a concentration of 100 μM. YOYO-1 was added at the lower concentration of 1 μM, because of its stronger fluorescence. Upon binding to DNA duplexes, the fluorescence intensity of the intercalators was increased. The ODNs (**1**+**2**+**3**) in the presence of YOYO-1 and EB showed spherical fluorescent assemblies with average diameters of 4.27 ± 0.45 μm and 6.29 ± 0.82 μm, respectively, (Figure 2B−D, $[ODN]_{total}$ = 20 μM, at 25 °C). It is clear that these intercalators are bound to spherical ODN assemblies.

Maeda et al. reported that the aggregation of colloid particles bearing DNA was induced by non-cross-linking DNA hybridization at high salt concentration.[22] It is probable that the aggregation of nucleospheres in Figure 2A,B,D is caused by the attractive force between the spheres at high salt concentration, although the absence of sticky ends on the surface of nucleospheres is not denied completely.

Note that the spherical assemblies also show fluorescence from the interior. It seems that the DNA assemblies are not hollow structures and have almost homogeneous density. As discussed in the previous paper,[19] ligated nucleospheres were resistant to enzymatic digestion by mung bean nuclease or exonuclease III, indicating the absence of single- or double-stranded terminals on the nucleosphere surface. Thus, it is probable that the nucleospheres are composed of a closed microgel-like structure or a multilayered shell (onion-like) structure.

On the other hand, a mixture of linear long λ-DNAs (48 000 bp) and YOYO-1 gave indistinct random structures in CLSM (Figure 2E). The addition of cetyltrimethylammonium bromide (CTAB) to this λ-DNA solution induced condensation of DNA, resulting in the formation of fluorescent globules (Figure 2F), as reported by Yoshikawa et al.[23] These results clearly show that three-way junctions tend to form spherical assemblies. This is further supported by the absence of such spherical assemblies for ternary ODN mixtures with two (**1**+**2**+**6**), one (**3**+**4**+**5**),

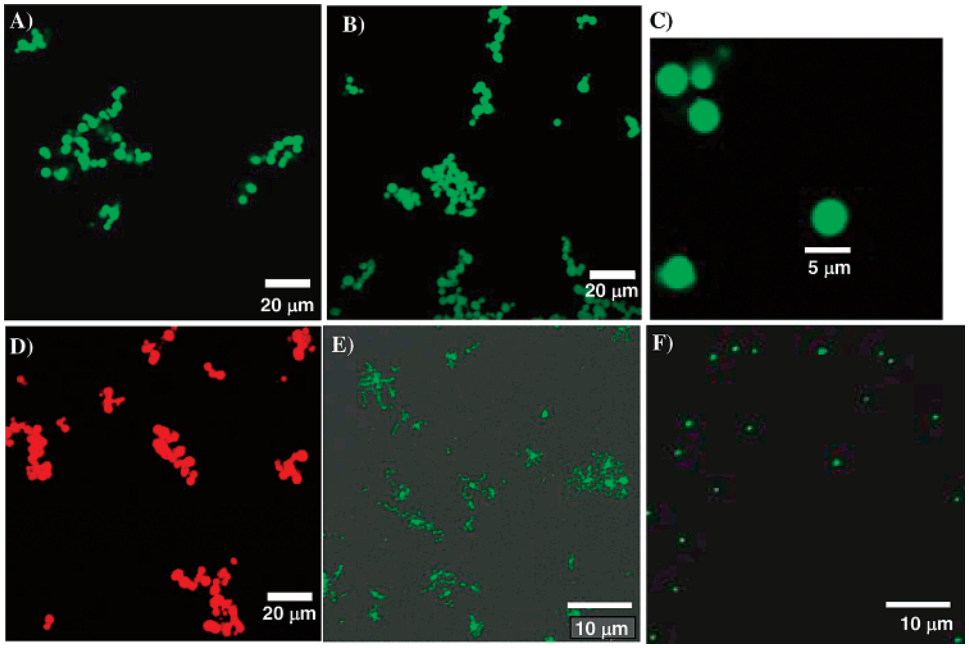


Figure 2. CLSM images of nucleospheres in 0.5 M NaCl aqueous solution at $[ODN]_{total} = 20\ \mu M$: (A) FAM-labeled nucleospheres (**1**+**2**^{FAM}+**3**), (B) nucleospheres (**1**+**2**+**3**) post-stained with YOYO-1 (1 μM), (C) magnified image of nucleospheres post-stained with YOYO-1 (1 μM), and (D) nucleospheres (**1**+**2**+**3**) post-stained with EB (100 μM). (E) λ-DNA ([base] = 600 μM) post-stained with YOYO-1 (1 μM). (F) λ-DNA ([base] = 600 μM) condensed by the addition of CTAB (1 mM). The sample is post-stained with YOYO-1 (1 μM).

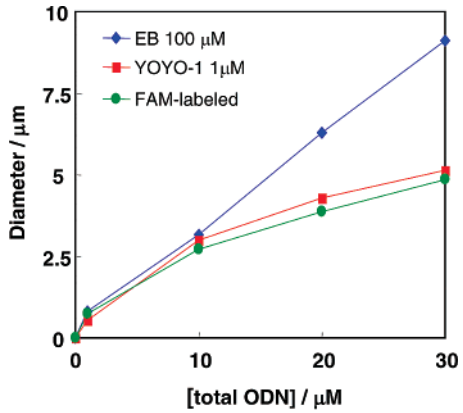


Figure 3. The effect of ODN concentration on the size of nucleospheres (**1**+**2**+**3**). Diameters of nucleospheres were determined by CLSM image.

and no sticky ends (**4**+**5**+**6**), which showed only homogeneous fluorescence images. Similarly, when two of the three strands (**1**, **2**, **3**) were mixed, spherical aggregates were not obtained. Therefore, it is apparent that the self-assembly of these spherical fluorescent spheres requires both three-way junctions and the full hybridization of complementary sticky ends.

Dependence of Nucleosphere Size on the Concentrations of ODN and Electrolyte. The size of nucleospheres as observed by TEM and dynamic light scattering (DLS) increased with the total concentration of ODNs. The DLS diameter of nucleospheres was 48.0 ± 10.4 nm at an ODN concentration of 1 μM, and it increased to 275.8 ± 66.8 nm at a higher ODN concentration of 5 μM.[19] Here, we investigated the concentration dependence by using CLSM. The average size of the nucleospheres obtained from CLSM images is shown in Figure 3 (concentration range of total ODN, 1−30 μM). The size of FAM-labeled nucleospheres (**1**+**2**^{FAM}+**3**) increased with the total ODN concentration, and reached an average size of 4.85 ± 1.42 μm ($[ODN]_{total}$ = 30 μM). The YOYO-1-adsorbed nucleospheres (**1**+**2**+**3**/YOYO-1) show almost similar concentration dependence (filled squares in Figure 3), supporting the

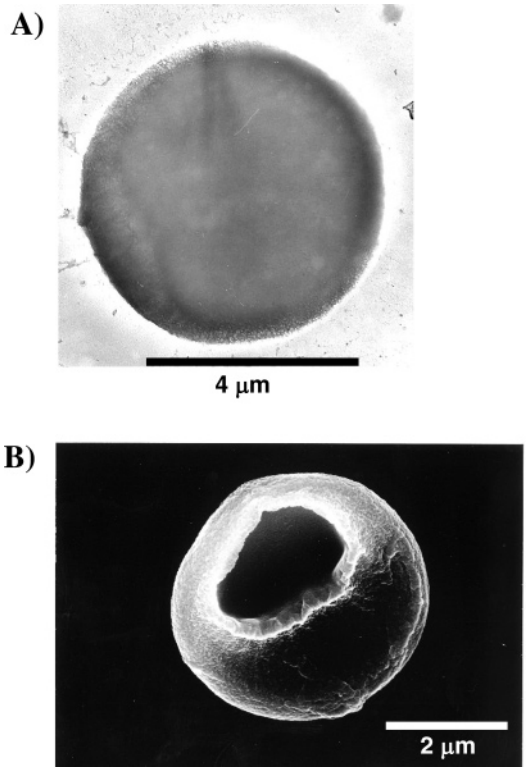


Figure 4. TEM (A) and SEM (B) images of nucleospheres (**1**+**2**+**3**) in 0.5 M NaCl aqueous solution. $[ODN]_{total} = 30\ \mu M$, [EB] = 100 μM.

suitability of these fluorescence-labeling techniques. The observed concentration dependence is consistent with the multilayered or closed microgel-like structure. On the other hand, the size of EB-stained nucleospheres (**1**+**2**+**3**/EB, [EB] = 100 μM) increased linearly and gave larger particles compared to those of the FAM- or YOYO-1-labeled samples. This may be caused by the existence of excess EBs.

TEM and SEM images of **1**+**2**+**3**/YOYO-1 assemblies (Figure 4, $[ODN]_{total}$ = 30 μM) revealed the formation of

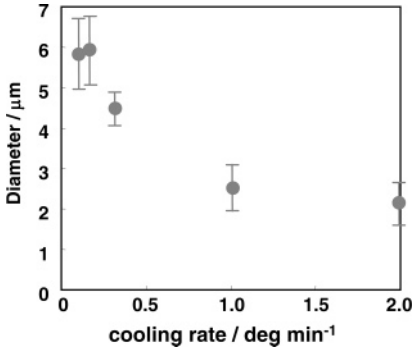


Figure 5. The effect of cooling rate on the size of nucleospheres (**1**+**2**+**3**) in the CLSM images. $[ODN]_{total} = 20\ \mu M$, [NaCl] = 0.5 M, [YOYO-1] = 1 μM. The error bar indicates the standard deviation of the average diameter of the nucleospheres.

spherical assemblies with a size of about 5−7 μm, which supports the CLSM observation. In the SEM image, a hollow structure with a thickness of ca. 200 nm was observed, which may reflect the drying of multilayered "onion-like" structures (thickness of deposited Pt, 7−9 nm).

The concentration of added electrolytes also affects the morphology of nucleospheres. CLSM of ODNs (**1**+**2**$^{\mathbf{FAM}}$+**3**, total 20 μM) in 0.5 M aqueous NaCl showed spherical nucleospheres with a size of about 3.9 μm (filled circles in Figure 3), whereas those in 0.1 M aqueous NaCl gave amorphous structures with a size of 1−2 μm (data not shown, at 25 °C). Further decrease in NaCl concentration to 0.05 M caused the disappearance of spherical structures, and a homogeneously fluorescent background was obtained.

The nucleospheres (**1**+**2**+**3**) give a double-step melting curve with $T_{m1} = 41$ °C and $T_{m2} = 56$ °C (in 0.5 M NaCl aq). The terms T_{m1} and T_{m2} correspond to the melting of sticky ends and three-way junctions, respectively. The melting temperatures (T_{m1}) of the nucleospheres (**1**+**2**+**3**) were lowered to 32 °C in 0.1 M NaCl (aq) and to 27 °C in 0.05 M NaCl (aq) (see Supporting Information). The absence of assemblies in 0.05 M aqueous NaCl suggests that hybridization of sticky ends does not fully occur at the temperature of CLSM observation (25 °C) because of the insufficient shielding of electrostatic repulsions among three-way junctions.

The Effect of Nucleosphere Construction Process on Size and Morphology. The thermal hybridization process also affected the size and morphology of the nucleospheres. In the above experiments, nucleospheres are formed by mixing equimolar ODNs (**1**, **2**, **3**) in 0.5 M aqueous NaCl, and then the mixture is once heated to 70 °C to completely dissociate the ODN aggregates. These mixtures are subsequently cooled to 10 °C spontaneously. The heating process is necessary to produce nucleospheres, since the mixing of equimolar ODNs (**1**, **2**, **3**) at 10 °C (NaCl, 0.5 M) only gave irregular structures. Similarly, irregular structures were obtained when equimolar mixture of ODNs (**1**+**2**+**3**) in 0.5 M aqueous NaCl (70 °C) was rapidly cooled to 10 °C. Thus, a slow cooling process is needed to self-assemble nucleospheres. Accordingly, the effect of cooling rate on the average size of nucleospheres is investigated (Figure 5). When ODN mixtures were cooled at constant rates of 1 and 2 °C min^{-1}, the average diameter of the nucleospheres was about 2 μm. Nanospheres with larger average diameter (ca. 4.27 μm) were obtained when the 70 °C sample was allowed to cool spontaneously (to 10 °C) at a slower rate (between 0.33 °C min^{-1} and 1 °C min^{-1}). On the other hand, larger assemblies with diameters of ca. 6 μm were obtained at much slower cooling rates of 0.17 and 0.11 °C min^{-1}.

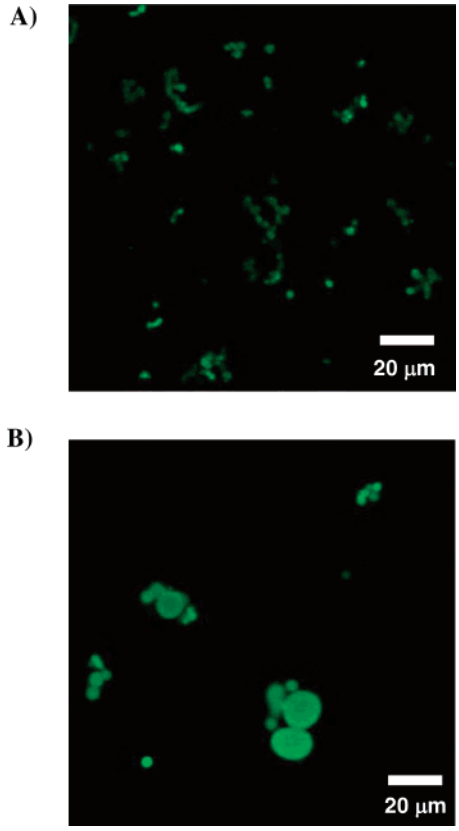


Figure 6. The effect of sticky-end sequences on the structure of nucleospheres: (A) ODNs **7**, **8**, and **9** (G_5C_5 sticky end), (B) ODNs **10**, **11**, and **12** ($(AT)_5$ sticky end). $[ODN]_{total} = 20\ \mu M$, [NaCl] = 0.5 M, [YOYO-1] = 1 μM.

The observed dependence of nucleosphere size on the cooling rate is ascribed to kinetic parameters such as time in that samples stay at a temperature around the T_m of sticky ends. This is confirmed by keeping the sample at 40 °C (near T_{m1}) for 3 h during the cooling process (from 70 °C to 10 °C, at 0.33 °C min^{-1}). The 40 °C-incubated sample showed nucleospheres with average diameters of 7.06 ± 1.71 μm, which is larger than those obtained without incubation (diameter, 4.23 ± 0.57 μm). These results suggest the possibility that nucleospheres were grown by an Ostwald ripening-type mechanism to form larger spheres.

Effect of Sticky-End Sequences on the Structure of Nucleospheres. The formation of nucleospheres with sticky ends of G_5C_5 (**7**+**8**+**9**) or $(AT)_5$ (**10**+**11**+**12**) was further examined. Figure 6A shows a CLSM image for equimolar ODNs **(7**+**8**+**9)** bearing G_5C_5 sticky ends ($[ODN]_{total} = 20\ \mu M$, [NaCl] = 0.5 M, spontaneously cooled sample). In the CLSM image, nucleospheres with a size of 2.10 ± 0.44 μm are abundantly seen, which are smaller than nucleospheres (**1**+**2**+**3**, A_5T_5 sticky ends) formed under the same conditions (4.27 ± 0.45 μm). The nucleospheres **(7**+**8**+**9)** showed a single-step melting curve with $T_m = 60$ °C, in contrast to the double-step curve with $T_m = 41$ and 56 °C observed for **1**+**2**+**3** (see Supporting Information). Under the spontaneous cooling conditions (i.e., cooling rate is not constant), samples are cooled faster at higher temperatures and slowly near or at room temperature. It limits the time that **7**+**8**+**9** stay around ca. 60 °C and results in a smaller nucleosphere size.

On the other hand, the self-assembly of ODNs (**10**+**11**+**12**) bearing $(AT)_5$ sticky ends ($[ODN]_{total} = 20\ \mu M$, [NaCl] = 0.5 M, spontaneously cooled sample) afforded nucleospheres with a polydispersed size of 5.78 ± 3.59 μm (Figure 6B). It was also observed that some irregular nucleospheres were fused

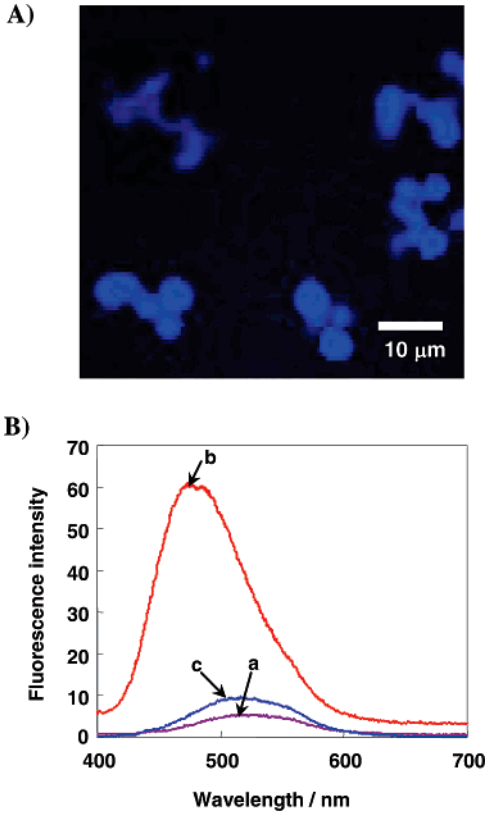
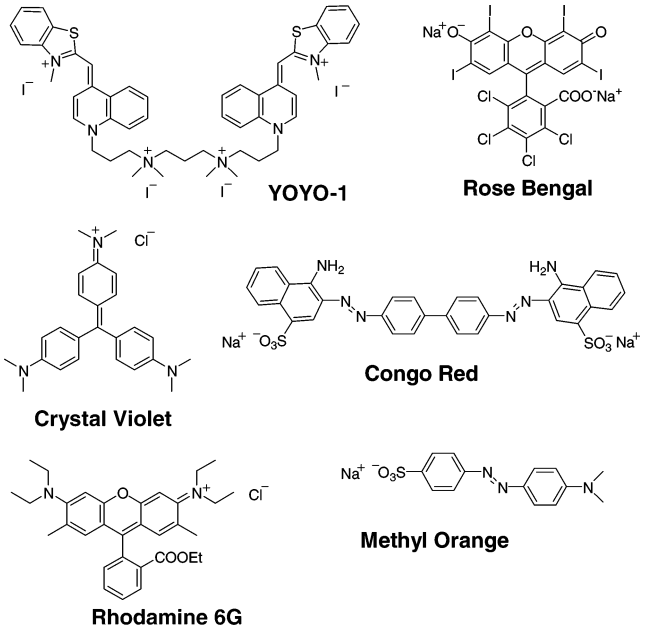


Figure 7. (A) CLSM image of nucleospheres constructed in the presence of 100 μM ANS. (B) Fluorescence spectra of ANS at 10 °C (a) in water, (b) in an aqueous solution of nucleospheres ($[ODN]_{total}$ = 20 μM, [NaCl] = 0.5 M), and (c) in an aqueous solution of salmon testes DNA ([base] = 600 μM, [NaCl] = 0.5 M).

together with the progress of time. Since the nucleospheres bearing $(AT)_5$ sticky ends have lower melting temperatures (T_m = 28 and 53 °C, see Supporting Information), it is probable that incomplete hybridization at $(AT)_5$ sticky ends causes such less controlled self-assembly at the temperature of CLSM observation (25 °C).

Polarity of the Interior of Nucleospheres. Nucleospheres (**1**+**2**+**3**) were prepared in the presence of polarity-sensitive fluorescent dye 1-anilinonaphthalene-8-sulfonate (ANS) ($[ODN]_{total}$ = 20 μM, [ANS] = 100 μM, 0.5 M aqueous NaCl). In CLSM images, blue fluorescent spheres with a size of 3.4−6.4 μm were abundantly seen, indicating that anionic ANS molecules were accumulated in nucleospheres (Figure 7A). When ANS was added to the solution of preformed nucleospheres, blue fluorescence spheres was not observed in CLSM, indicating that ANS was not trapped spontaneously to the preformed nucleospheres. Fluorescence spectra of ANS in aqueous nucleospheres (**1**+**2**+**3**) exhibited an intense peak at 478 nm (Figure 7B(b)). As compared with ANS in pure water (Figure 7B, a), the maximum of emission peak is blue-shifted, and the intensity is increased 8-fold. On the other hand, ANS in aqueous salmon testes DNA shows fluorescence spectra with intensity slightly increased compared to that in pure water (Figure 7B-(c)). These results indicate that the interior of nucleospheres provides medium micropolarity. The amount of ANS included in nucleospheres was estimated by measuring the ANS concentration that remained in the supernatant after centrifugation (10 000 rpm, 10 min). As a result, 4.7 mol % of added ANS was included in the nucleospheres ($[ODN]_{total}$ = 20 μM). This value is significantly greater than would be expected based solely on statistical considerations, since the volume % represented by the spheres is calculated to be about 0.03 vol %. It

Chart 1. Structures of Dyes Used in This Study.



indicates that anionic dyes can bind to nucleospheres probably because of the high ionic strength, which may shield the electrostatic repulsion between ANS and nucleospheres.

The peak wavelength of ANS fluorescence is correlated with the polarity scale (E_T^N value) of solvent.[24] According to the relationship, the polarity inside nucleospheres corresponds to an E_T^N value of 0.72, which is between that of methanol (E_T^N = 0.76) and ethanol (E_T^N = 0.65). ANS is bound to the medium polar interior of nucleospheres, and this is a unique feature not available from linear DNA strands.

Inclusion of Dyes to Nucleospheres and Their Controlled Release. The interiors of nucleospheres possess medium micropolarity, which would render them as a host for hydrophobic molecules. The interaction of various cationic and anionic dyes (Chart 1) was investigated by mixing ODNs (**1**+**2**+**3**) and dyes. Nucleospheres ($[ODN]_{total}$ = 10 μM) were prepared by heating and cooling in 0.5 M aqueous NaCl. Figure 8A shows a CLSM image of nucleospheres in the presence of Rose Bengal. It is seen that anionic Rose Bengal is adsorbed to nucleospheres, similarly to the case of ANS.

The amount of dyes included in nucleospheres can be quantitatively estimated by measuring the concentration of dyes in the supernatant, after centrifugation of the dye-including nucleospheres (Figure 8B). Cationic dyes Rhodamine 6G, Crystal Violet, and YOYO-1 all bind to nucleospheres. Note that Rhodamine 6G and Crystal Violet do not intercalate to linear salmon testes DNA at [base] = 250 μM (see Supporting Information). Rose Bengal and Congo Red, highly hydrophobic anionic dyes, strongly bind to nucleospheres, whereas no binding was observed for Methyl Orange. When the dye-including nucleospheres were centrifuged to separate them from bulk solutions and were exposed to fresh 0.5 M aqueous NaCl for 48 h, leakage of dyes from the nucleospheres was hardly observed.

Figure 9 shows the fraction of bound dyes in the course of heating the aqueous mixture. Anionic Rose Bengal was effectively released from nucleospheres at temperatures near 30 °C, and it was completely released at 60 °C (Figure 9a). It is probable that cooperative destruction of nucleospheres near the melting temperature caused the drastic release of Rose Bengal from nucleospheres. Cationic Crystal Violet is similarly

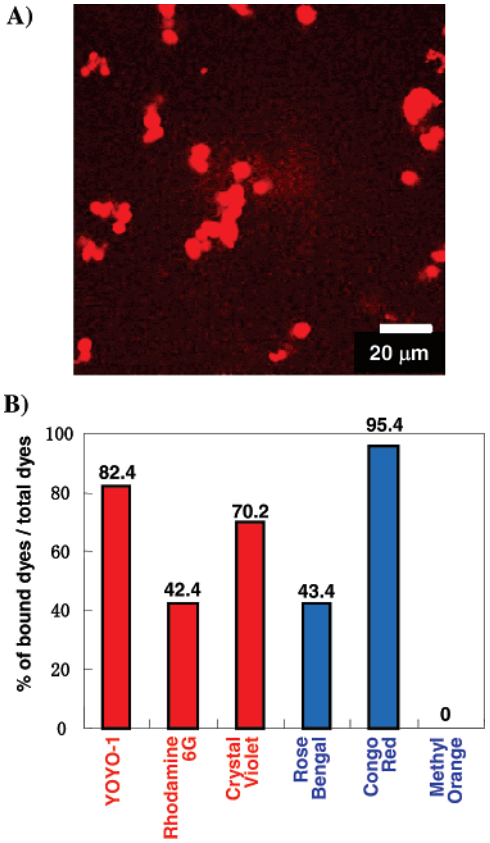


Figure 8. Inclusion of dyes to nucleospheres. (A) CLSM image of nucleospheres formed in the presence of 100 μM Rose Bengal. (B) The proportion of dyes bound on nucleospheres to the total amount of dyes.

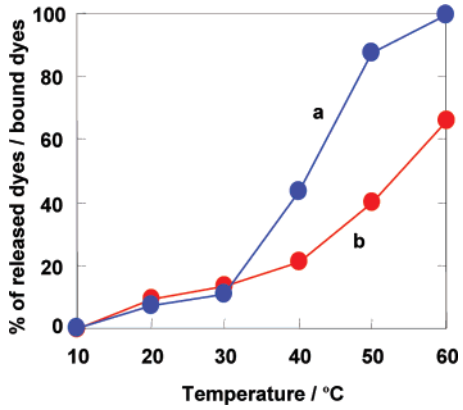


Figure 9. The effect of temperature on the controlled release of Rose Bengal (a) and Crystal Violet (b) from nucleospheres.

released from nucleospheres above 30 °C, but show higher affinity to ODNs at higher temperatures. It may be that hydrophobic ion pairs formed between ODNs and Crystal Violet are stable even at higher temperatures.

Enzymatic control on the release of bound dyes was further investigated. To aqueous dispersions of dye-adsorbed nucleospheres ([ODN] = 10 μM, [dye] = 100μM, [NaCl] = 0.5M) was added aqueous endonuclease DNase I (10 unit). After keeping the mixtures for 30 min, they were centrifuged. The concentration of free dyes in the supernatant was determined by UV−vis absorption intensities. As shown in Figure 10, the cationic dyes (YOYO-1, Rhodamine 6G, Crystal Violet) were efficiently released from nucleospheres upon the addition of DNase I, indicating that the enzymatic digestion of nucleospheres induced the release of electrostatically bound dyes. This

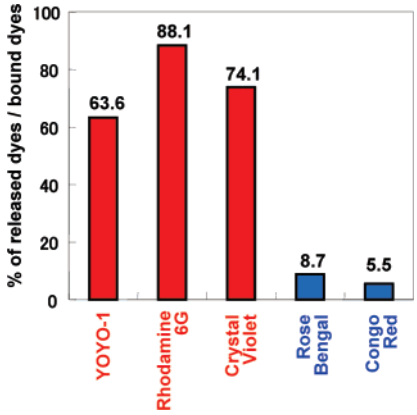


Figure 10. The proportion of dyes released by the digestion of nucleospheres with DNase I.

is supported by CLSM, where spherical structures are destroyed upon the addition of DNase I. In contrast, the anionic dyes (Congo Red, Rose Bengal) were hardly released to the bulk water phase. The CLSM image of anionic dye-included nucleospheres was barely affected by the addition of DNase I. It seems that the activity of DNase I was repressed by the presence of anionic dyes, since the activity of DNase I for the digestion of salmon testes DNA was reduced to about 16% of that in the absence of dye in the presence of Rose Bengal (100 μM). These results indicate the potential of nucleospheres that function as thermally responsive and biodegradable carriers for hydrophobic drugs and biomolecules.

Conclusion

CLSM observations clearly indicate that DNA three-way junctions bearing complementary sticky ends self-assemble into cell-sized nucleospheres in water. The size and morphology of the nucleospheres were affected by ODN concentration, salt strength, cooling rate, and the sequence of the self-complementary sticky ends. The DNA assemblies have an almost homogeneous density, rather than forming hollow structures. The interior of the nucleospheres gave medium micropolarity, which is comparable to that of ethanol. Hydrophobic dyes are included in the inside of nucleospheres regardless of the charge. They are released in response to the thermal melting of duplexes or by the enzymatic digestion of nucleospheres. The self-assembly of spherical DNA is very simple, and it would be widely applicable to the design of biodegradable carriers for hydrophobic drugs and biomolecules. Their use as scaffolds for preparing functional materials is also envisaged.

Acknowledgment. The present work is supported by a Grant-in-Aid for the 21st Century COE Program, "Functional Innovation of Molecular Informatics" from the Ministry of Education, Culture, Sports, Science and Technology, by a Grant-in-Aid for Young Scientists (B) (15750147), and by a Grant-in-Aid for Exploratory Research (18655073) from the Japan Society for the Promotion of Science. We also thank Professor I. Hamachi (Kyoto University) for the use of the Carl Zeiss LSM 510 and his useful suggestions.

Supporting Information Available. Melting curves of nucleospheres in various concentration of aqueous NaCl solution (Figure S1A), melting curves of nucleospheres bearing various sticky ends (Figure S1B), and intercalation behavior of cationic dyes with salmon testes DNA (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Branden, C.; Tooze, J. *Introduction to Protein Structure*, 2nd ed.; Garland Publishing: New York, 1999.

(2) (a) Olson, A. J.; Bricogne, G.; Harrison, S. C. *J. Mol. Biol.* **1983**, *171*, 61. (b) Grimes, J. M.; Burroughs, J. N.; Gouet, P.; Diprose, J. M.; Malby, R.; Ziéntara, S.; Mertens, P. P. C.; Stuart, D. I. *Nature* **1998**, *395*, 470.

(3) (a) Kunitake, T.; Okahata, Y. *J. Am. Chem. Soc.* **1977**, *99*, 3890. (b) Kunitake, T. *Angew. Chem., Int. Ed.* **1992**, *31*, 709.

(4) Kimizuka, N. *Curr. Opin. Chem. Biol.* **2003**, *7*, 702.

(5) (a) Wyler, R.; de Mendoza, J.; Rebek, J., Jr. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1699. (b) Rebek, J., Jr. *Chem. Soc. Rev.* **1996**, 255.

(6) (a) Sun, W. Y.; Yoshizawa, M.; Kusukawa, T.; Fujita, M. *Curr. Opin. Chem. Biol.* **2002**, *6*, 757. (b) Fujita, M.; Tominaga, M.; Hori, A.; Therrien, B. *Acc. Chem. Res.* **2005**, *38*, 369.

(7) (a) Jenekhe, S. A.; Chen, X. L. *Science* **1998**, *279*, 1903. (b) Jenekhe, S. A.; Chen, X. L. *Macromolecules* **2000**, *33*, 4610.

(8) (a) Velonia, K.; Rowan, A. E.; Nolte, R. J. M. *J. Am. Chem. Soc.* **2002**, *124*, 4224. (b) Boerakker, M. J.; Hannink, J. M.; Bomans, P. H. H.; Frederik, P. M.; Nolte, R. J. M.; Meijer, E. M.; Sommerdijk, N. A. J. M. *Angew. Chem., Int. Ed.* **2002**, *41*, 4239. (c) Boerakker, M. J.; Botterhuis, N. E.; Bomans, P. H. H.; Frederik, P. M.; Meijer, E. M.; Nolte, R. J. M.; Sommerdijk, N. A. J. M. *Chem.—Eur. J.* **2006**, *12*, 6071.

(9) (a) Dähne, L.; Leporatti, S.; Donath, E.; Möhwald, H. *J. Am. Chem. Soc.* **2001**, *123*, 5431. (b) Gao, C.; Leporatti, S.; Moya, S.; Donath, E.; Möhwald, H. *Chem.—Eur. J.* **2003**, *9*, 915. (c) Shchukin, D. G.; Köhler, K.; Möhwald, H.; Sukhorukov G. B. *Angew. Chem., Int. Ed.* **2005**, *44*, 3310. (d) Ma, Y.; Dong, W.-F.; Hempenius, M. A.; Möhwald, H.; Vancso, G. J. *Nat. Mater.* **2006**, *5*, 724.

(10) For reviews, see (a) Seeman, N. C. *Acc. Chem. Res.* **1997**, *30*, 357. (b) Seeman, N. C. *Angew. Chem., Int. Ed.* **1998**, *37*, 3220. (c) Storhoff, J. J.; Mirkin, C. A. *Chem. Rev.* **1999**, *99*, 1849. (d) Niemeyer, C. M. *Curr. Opin. Chem. Biol.* **2000**, *4*, 609. (e) Niemeyer, C. M. *Angew. Chem., Int. Ed.* **2001**, *40*, 4128. (f) Niemeyer, C. M. *Angew. Chem., Int. Ed.* **2002**, *41*, 3779. (g) Seeman, N. C. *Nature* **2003**, *421*, 427. (h) Seeman, N. C. *Biochemistry* **2003**, *42*, 7259. (i) Gothelf, K. V.; LaBean, T. H. *Org. Biomol. Chem.* **2005**, *3*, 4023. (j) Feldkamp, U.; Niemeyer, C. M. *Angew. Chem., Int. Ed.* **2006**, *45*, 2.

(11) (a) Chen, J.; Seeman, N. C. *Nature* **1991**, *350*, 631. (b) Zhang, Y.; Seeman, N. C. *J. Am. Chem. Soc.* **1994**, *116*, 1661. (c) Mao, C.; Sun, W.; Seeman, N. C. *Nature* **1997**, *386*, 3655. (d) Nilsen, T. W.; Grayzel, J.; Prensky, W. *J. Theor. Biol.* **1997**, *187*, 273. (e) Wang, J.; Jiang, M.; Nilsen, T. W.; Getts, R. C. *J. Am. Chem. Soc.* **1998**, *120*, 8281. (f) Winfree, E.; Liu, F.; Wenzler L. A.; Seeman N. C. *Nature* **1998**, *398*, 539. (g) Scheffer, M.; Dorenbeck, A.; Jordan, S.; Wüstefeld, M.; von Kiedrowski, G. *Angew. Chem., Int. Ed.* **1999**, *38*, 3312. (h) LaBean, T. H.; Yan, H.; Kopatsch, J.; Liu, F. R.; Winfree, E.; Reif, J. H.; Seeman, N. C. *J. Am. Chem. Soc.* **2000**, *122*, 1848. (i) Shih, W. M.; Quispe, J. D.; Joyce, G. F. *Nature* **2004**, *427*, 618. (j) Mitchell, J. C.; Harris, J. R.; Malo, J.; Bath, J.; Turberfield, A. J. *J. Am. Chem. Soc.* **2004**, *126*, 16342. (k) Rothemund, P. W. K.; Nkodo, A. E.; Papadakis, N.; Kumar, A.; Fygenson, D. K.; Winfree, E. *J. Am. Chem. Soc.* **2004**, *126*, 16344. (l) Goodman, R. P.; Schaap, I. A. T.; Tardin, C. F.; Erben, C. M.; Berry, R. M.; Schmidt, C. F.; Turberfield, A. J. *Science* **2005**, *310*, 1661.

(12) (a) Mao, C.; LaBean, T. H.; Reif, J. H.; Seeman, N. C. *Nature* **2000**, *407*, 493. (b) Yan, H.; Feng, L.; LaBean, T. H.; Reif, J. H. *J. Am. Chem. Soc.* **2003**, *125*, 14246.

(13) (a) Mao, C.; Sun, W.; Shen, Z.; Seeman, N. C. *Nature* **1999**, *397*, 144. (b) Yurke, B.; Turberield, A. J.; Mills, A. P., Jr.; Simmel, F. C.; Neumann, J. L. *Nature* **2000**, *406*, 605. (c) Yan, H.; Zhang, X.; Shen, Z.; Seeman, N. C. *Nature* **2002**, *415*, 62. (d) Seelig, G.; Yurke, B.; Winfree E. *J. Am. Chem. Soc.* **2006**, *128*, 12211.

(14) (a) Cao, R.; Gu, Z.; Hsu, L.; Patterson, G. D.; Armitage, B. A. *J. Am. Chem. Soc.* **2003**, *125*, 10250. (b) Cao, R.; Gu, Z.; Patterson, G. D.; Armitage, B. A. *J. Am. Chem. Soc.* **2004**, *126*, 726.

(15) (a) Mirkin, C. A.; Letsinger, R. L.; Mucic, R. C.; Storhoff, J. J. *Nature* **1996**, *382*, 607. (b) Alivisatos, A. P.; Johnsson, K. P.; Peng, X.; Wilson, T. E.; Loweth, C. J.; Bruchez, M. P., Jr.; Schultz, P. G. *Nature* **1996**, *382*, 609. (c) Loweth, C. J.; Caldwell, W. B.; Peng, X.; Alivisatos, A. P.; Schultz, P. G. *Angew. Chem., Int. Ed.* **1999**, *38*, 1808. (d) Le, J. D.; Pinto, Y.; Seeman, N. C.; Musier-Forsyth, K.; Taton, T. A.; Kiehl, R. A. *Nano Lett.* **2004**, *4*, 2343.

(16) (a) Niemeyer, C. M.; Bürger, W.; Peplies, J. *Angew. Chem., Int. Ed.* **1998**, *37*, 2265. (b) Niemeyer, C. M.; Adler, M.; Gao, S.; Chi, L. *Angew. Chem., Int. Ed.* **2000**, *39*, 3056. (c) Niemeyer, C. M.; Koehler, J.; Wuerdemann, C. *ChemBioChem* **2002**, *3*, 242. (d) Liu, Y.; Lin, C.; Li, H. Yan, H. *Angew. Chem., Int. Ed.* **2005**, *44*, 2. (e) Park, S. H.; Yin, P.; Liu, Y.; Reif, J. H.; LaBean, T. H.; Yan, H. *Nano Lett.* **2005**, *5*, 729.

(17) (a) Ohya, Y.; Yabuki, K.; Komatsu, M.; Ouchi, T. *Polym. Adv. Technol.* **2000**, *11*, 845. (b) Ohya, Y.; Yabuki, K.; Hashimoto, M.; Nakajima, A.; Ouchi T. *Bioconjugate Chem.* **2003**, *14*, 1057.

(18) (a) Matsuura, K.; Hibino, M.; Yamada, Y.; Kobayashi, K. *J. Am. Chem. Soc.* **2001**, *123*, 357. (b) Akasaka, T.; Matsuura, K.; Kobayashi, K. *Bioconjugate Chem.* **2001**, *12*, 776. (c) Matsuura, K.; Hibino, M.; Ikeda, T.; Yamada, Y.; Kobayashi, K. *Chem.—Eur. J.* **2004**, *10*, 352. (d) Yamada, Y.; Matsuura, K.; Kobayashi, K. *Bioorg. Med. Chem.* **2005**, *13*, 1913.

(19) Matsuura, K.; Yamashita, T.; Igami, Y.; Kimizuka, N. *Chem. Commun.* **2003**, 376.

(20) (a) Kim, K.; Masumoto, K.; Matsuura, K.; Kimizuka, N. *Chem. Lett.* **2006**, *35*, 486. (b) Kim, K.; Matsuura, K.; Kimizuka, N. *Bioorg. Med. Chem.* **2007**, *15*, 4311.

(21) (a) Yoshikawa, K.; Matsuzawa, Y. *Physica D* **1995**, *84*, 220. (b) Yoshikawa, K.; Matsuzawa, Y. *J. Am. Chem. Soc.* **1996**, *118*, 929.

(22) Sato, K.; Hosokawa, K.; Maeda, M. *J. Am. Chem. Soc.* **2003**, *125*, 8102.

(23) (a) Mel'nikov, S. M.; Sergeyev, V. G.; Yoshikawa, K. *J. Am. Chem. Soc.* **1995**, *117*, 2401. (b) Mel'nikov, S. M.; Sergeyev, V. G.; Yoshikawa, K. *J. Am. Chem. Soc.* **1995**, *117*, 9951.

(24) Hayashida, O.; Ono, K.; Hisaeda, Y.; Murakami, Y. *Tetrahedron* **1995**, *51*, 8423.

BM070357W